Bioactivity Profiling of Environmental Chemicals in the EPA's ToxCast Program

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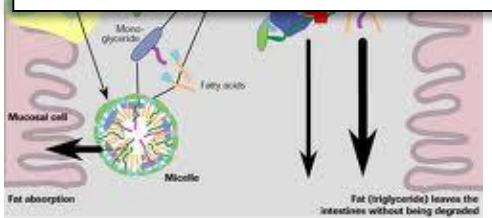
Problem Statement

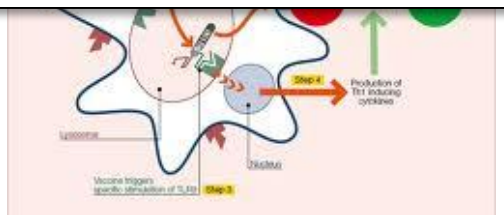
Too many chemicals to test with standard animal-based methods

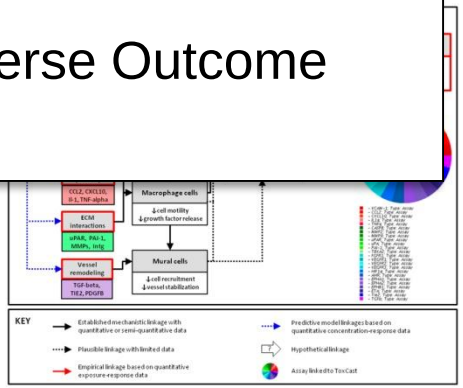
- Cost, time, animal welfare

Need for better mechanistic data

- Determine human relevance
- What is the Mode of Action (MOA) or Adverse Outcome Pathway (AOP)?







1996 Legislative Mandate

1996 Federal Food, Drug and Cosmetic Act, section 408(p)

Requires the U. S. EPA to develop a screening program using appropriate validated test systems and other scientifically relevant methods to determine whether certain substances may have an effect in humans that is similar to an effect produced by a naturally occurring estrogen, or other such endocrine effect as the Administrator may designate.

1996 Safe Drinking Water Act Amendments, section 1457

Testing of chemical substances that may be found in sources of drinking water, if substantial human populations may be exposed.

1998 Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC)

EDSTAC Key Recommendations:

- Expand Protection to Include Human Health and Wildlife
- Include Estrogen, Androgen and Thyroid Pathways
- Develop a Two-Tiered Screening and Testing Program:

EDSTAC Conceptual Framework:

Tier 1 Screening for *Potential* to Interact

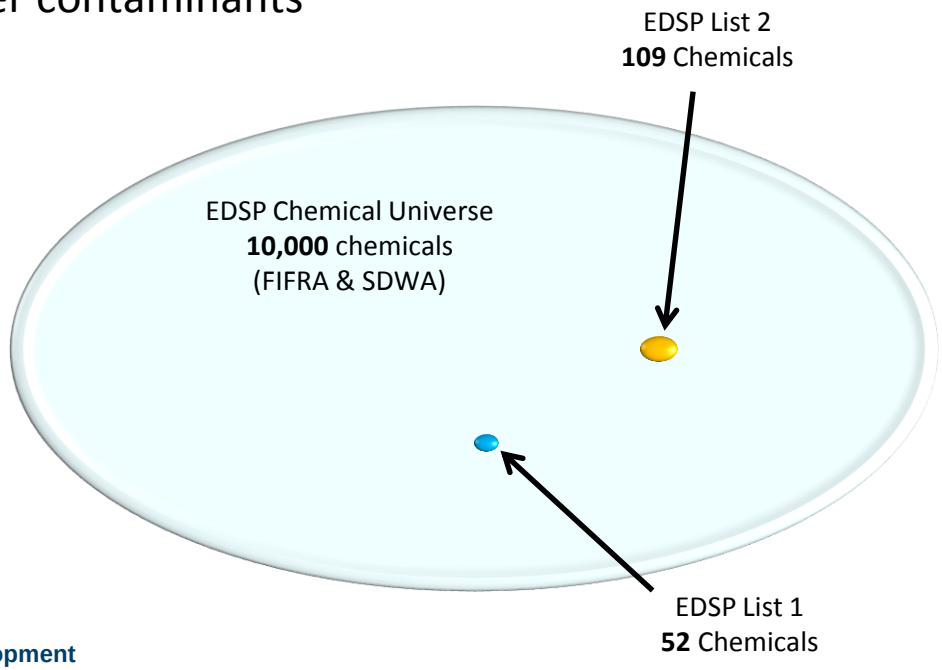
Potential to interact with the estrogen, androgen or thyroid hormone systems

Tier 2 Testing to determine Interaction with the endocrine system

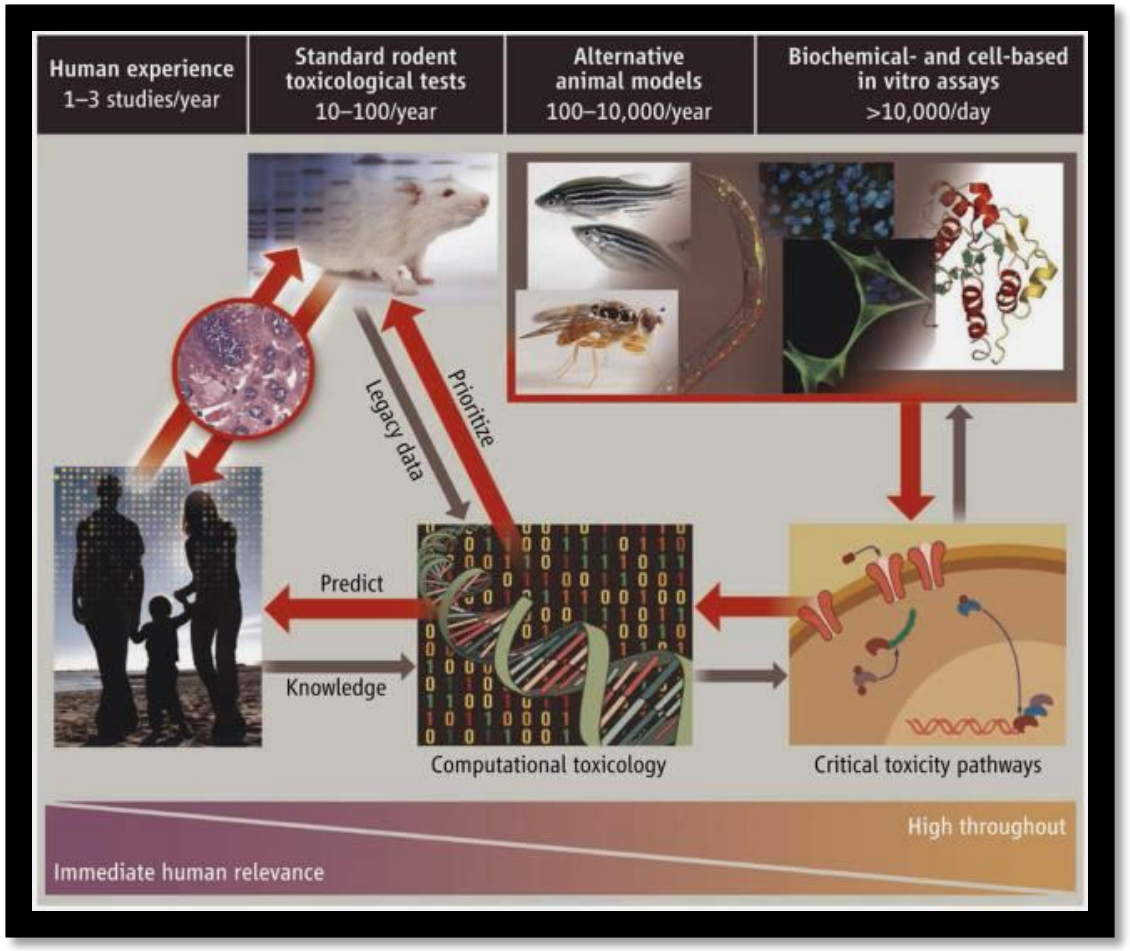
If endocrine-mediated adverse effects then quantify dose-response relationship

EDSP Implementation

- EDSP List 2 Chemicals
  - Draft EDSP List 2 chemicals for Tier 1 screening released (2010)
  - EPA issued revised EDSP List 2 with 109 chemicals (2013)
    - Selection based on registration review schedule of 41 pesticidal chemicals and 68 drinking water contaminants



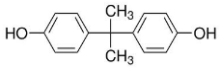
Tox21 Vision: Transforming Toxicity Testing



National Center for Advancing Translational Sciences (NCATS)
http://www.ncats.nih.gov/

SOURCE: Collins, Gray and Bucher (2008) Toxicology. Transforming environmental health protection. Science 319: 906

Toxicity Pathways



Exposure

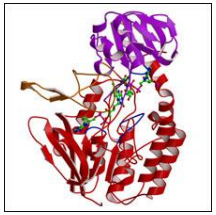
Tissue Dose

Biologic Interaction

Perturbation

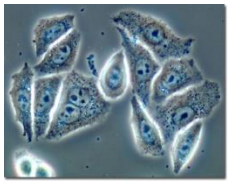
Cellular assays

Biochemical assays



Biologic Inputs

Normal Biologic Function



Cellular assays

Early Cellular Changes

Adaptive Stress Responses

Cell Injury

Cellular assays

Traditional animal toxicology

Morbidity and Mortality

When perturbations are sufficiently large or when the host is unable to adapt because of underlying nutritional, genetic, disease, or life-stage status, biologic function is compromised, and this leads to toxicity and disease.

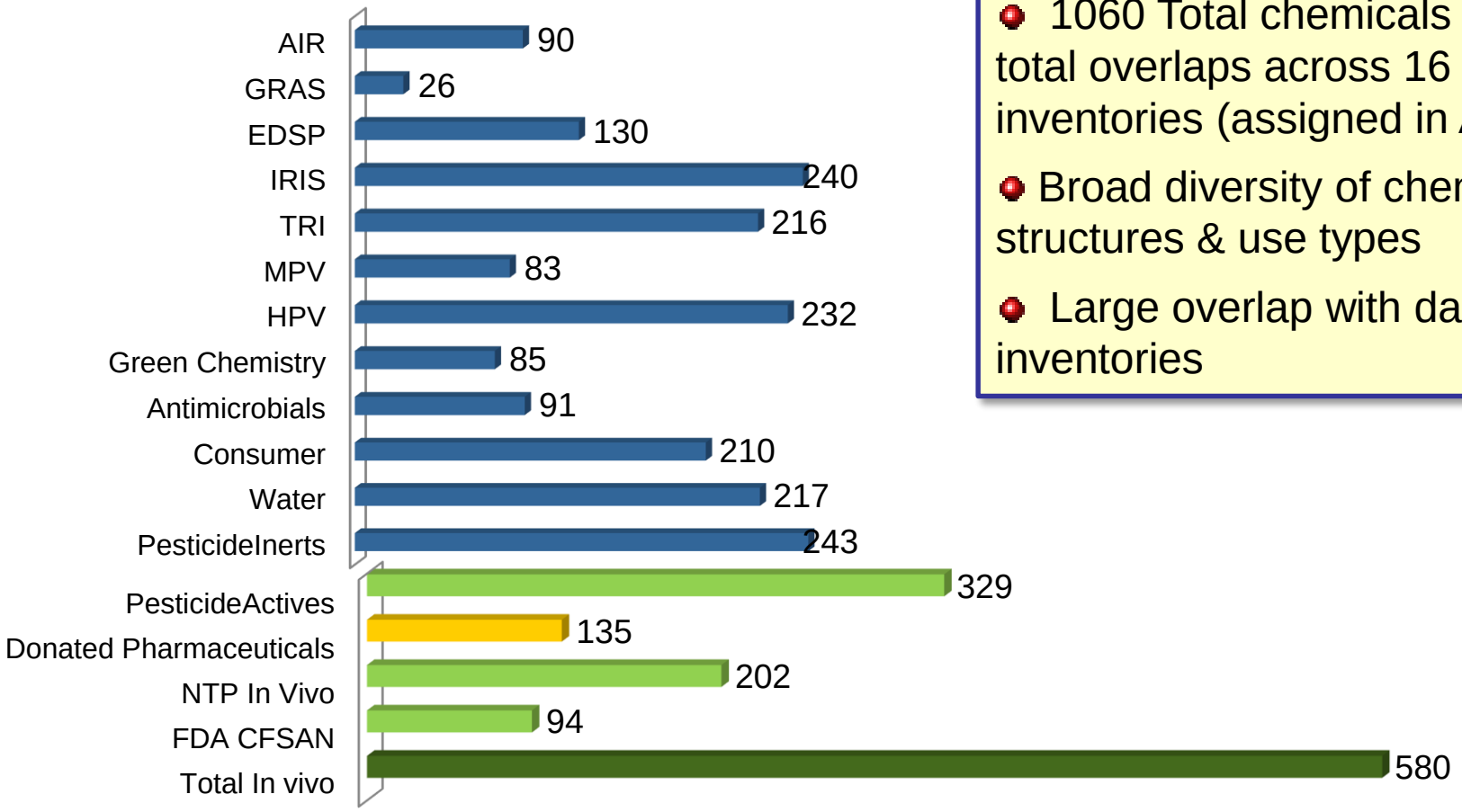
Toxicity Testing in the Twenty-first Century: A Vision and a Strategy, NRC, 2007

ToxCast/Tox21 Overall Strategy

- Identify targets or pathways linked to toxicity (AOP focus)
- Identify/develop high-throughput assays for these targets or pathways
- Develop predictive systems models:
  - *in vitro/in silico → in vivo*
- Use predictive models (qualitative):
  - Prioritize chemicals for targeted testing
  - Suggest / distinguish possible AOP / MOA for chemicals
- *High-throughput Exposure Predictions (ExpoCast)*
- *High-throughput Risk Assessments (quantitative)*

ToxCast PhI&PhII chemicals:

Spanning diverse inventories of EPA interest

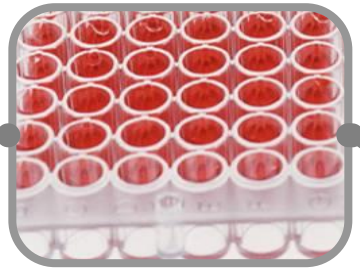


- 1060 Total chemicals → 2806 total overlaps across 16 diverse inventories (assigned in ACToR)
- Broad diversity of chemical structures & use types
- Large overlap with data-rich inventories

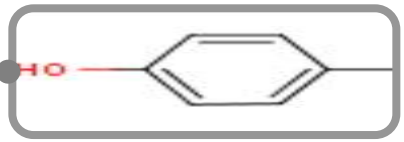
High-Throughput Screening 101 (HTS)

United States Environmental Protection Agency

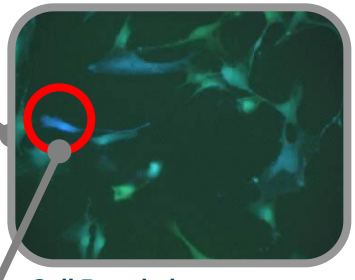
Robots



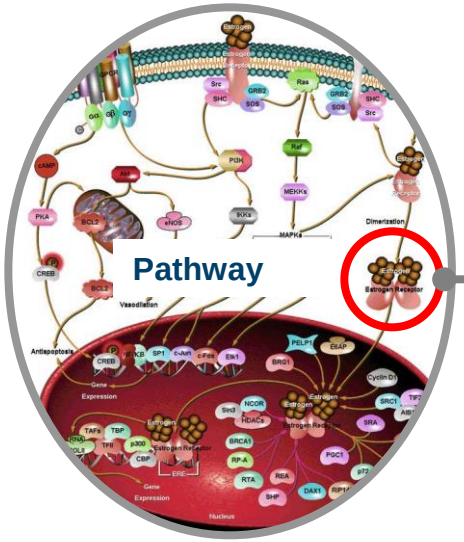
96-, 384-, 1536 Well Plates



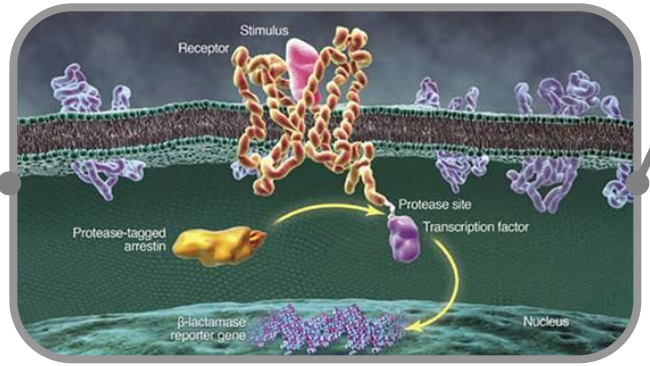
Chemical Exposure



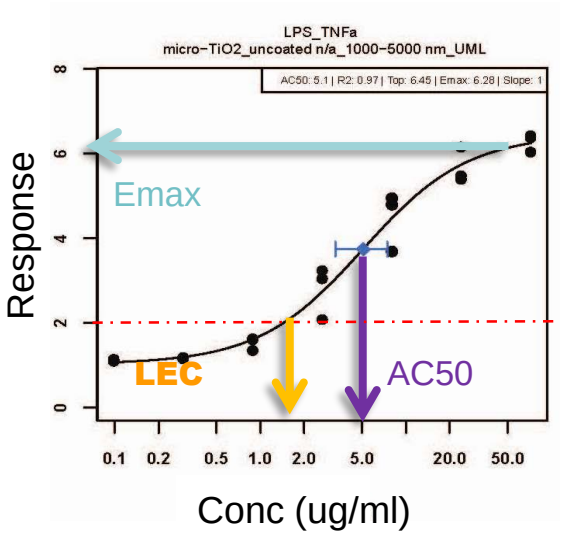
Cell Population



Pathway



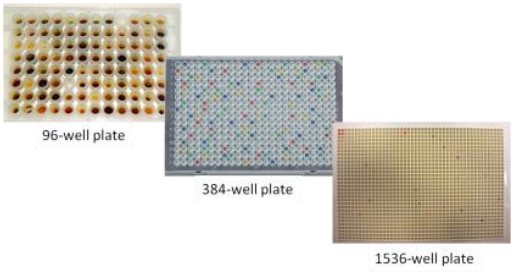
Target Biology (e.g., Estrogen Receptor)



Assay Selection Strategy

- Lack of extensive list of defined toxicity pathways/targets required broad approach
- Several rounds of solicitations for broad ranges of assays covering target gene families, critical pathways, toxicity phenotypes, complex cell culture systems, gene expression, developmental pathways
- Required:
  - Ability to efficiently screen thousands of chemicals
  - Existing, *validated* assays
  - Quality Assurance/Quality Control program

ToxCast Assays (>700 endpoints)



Assay Provider
ACEA
Apredica
Attagene
BioReliance
BioSeek
CeeTox
CellzDirect
Tox21/NCATS
NHEERL MESC
NHEERL Zebrafish
NovaScreen (Perkin Elmer)
Odyssey Thera
Vala Sciences

Biological Response
cell proliferation and death
cell differentiation
Enzymatic activity
mitochondrial depolarization
protein stabilization
oxidative phosphorylation
reporter gene activation
gene expression (qNPA)
receptor binding
receptor activity
steroidogenesis

Target Family
response Element
transporter
cytokines
kinases
nuclear receptor
CYP450 / ADME
cholinesterase
phosphatases
proteases
XME metabolism
GPCRs
ion channels

Assay Design
viability reporter
morphology reporter
conformation reporter
enzyme reporter
membrane potential reporter
binding reporter
inducible reporter

Readout Type
single
multiplexed
multiparametric

Cell Format
cell free
cell lines
primary cells
complex cultures
free embryos

Species
human
rat
mouse
zebrafish
sheep
boar
rabbit
cattle
guinea pig

Tissue Source

Lung	Breast
Liver	Vascular
Skin	Kidney
Cervix	Testis
Uterus	Brain
Intestinal	Spleen
Bladder	Ovary
Pancreas	Prostate
Inflammatory	Bone

Detection Technology
qNPA and ELISA
Fluorescence & Luminescence
Alamar Blue Reduction
Arrayscan / Microscopy
Reporter gene activation
Spectrophotometry
Radioactivity
HPLC and HPEC
TR-FRET

List of assays and related information at: **http://www.epa.gov/ncct/**

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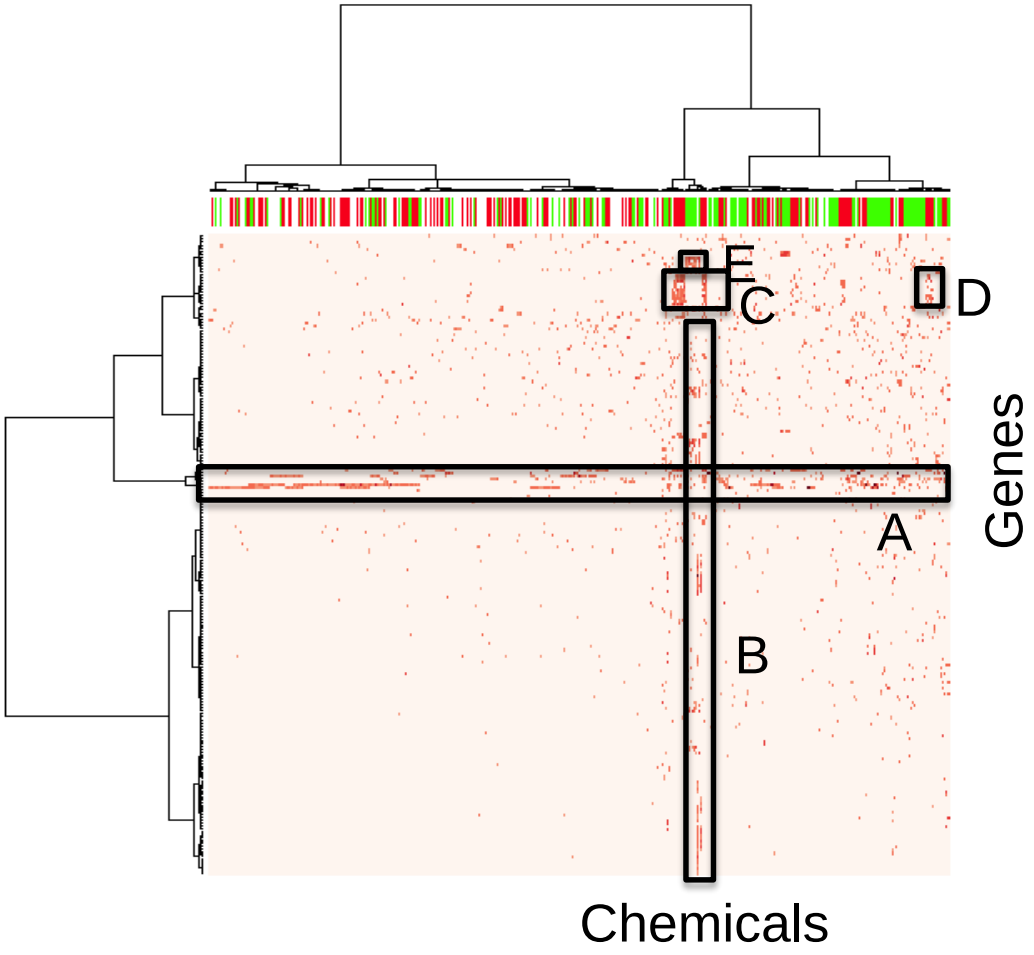
Gene Score – Summarize Effects

- How to summarize 1000s of chemicals x 100s of assays?
- Potency: -log(AC50) for each assay
- Annotation of assays by “Gene”
- Gene Score = mean potency across all assays for a gene

Broad look at Genes x Chemicals



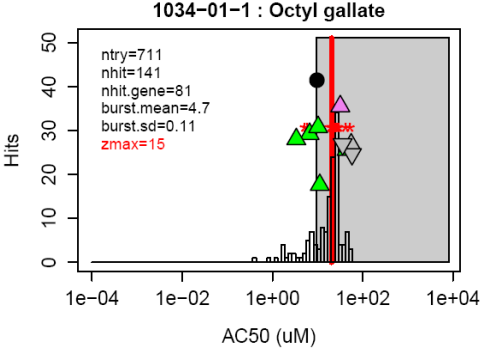
(A) Most promiscuous genes (COL3A1, SAA1, PTGER2, CYP2C19, NFE2L2, CYP27B1, H2AFX, NR1I2/PXR)

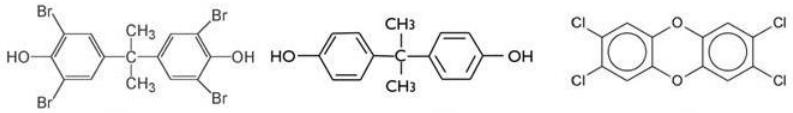
(B) Most promiscuous chemicals (Mancozeb, Titanium chelator, Maneb, Raloxifene, Imazalil, Chlorpromazine, Prochloraz, SSR150106, SSR146977)

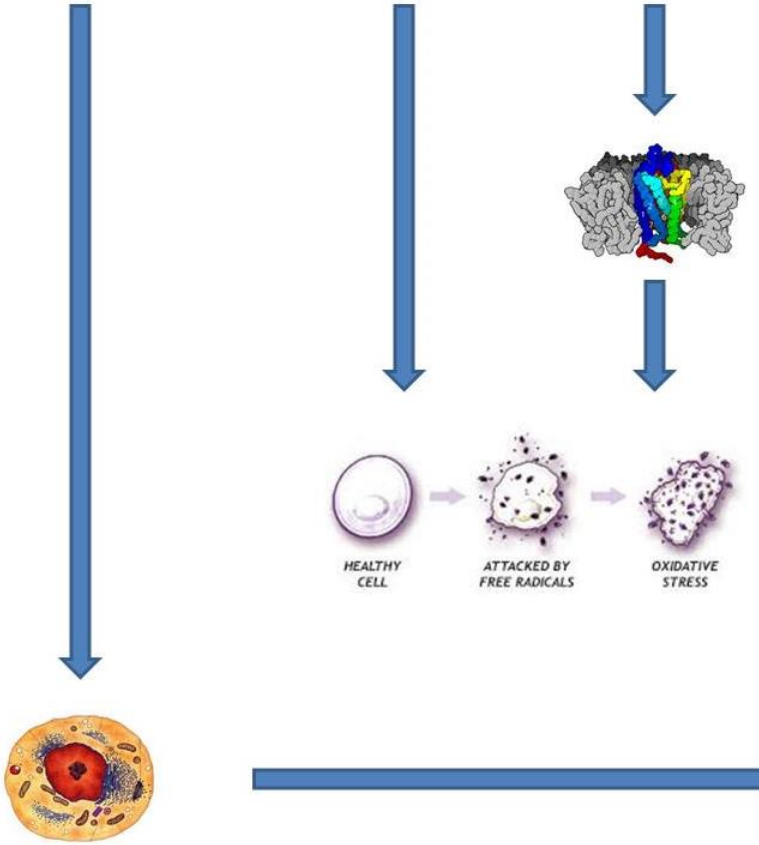
(C, D) CYP-450 genes and conazoles

(E) pharmaceuticals and drug targets: CHRM2, SLC6A3, HRH2, ADRA2C, HTR2C, HTR7

Significance of In Vitro Effects







Assay Target Class

Assessment

Molecular Target

EDC
Acetylcholinesterase Inhibition
Ion channel blocker
Genotoxicity

→ AOP Assessment
Targeted testing

Cell Stress Mediated

Oxidative stress
Membrane disruption
Nucleophiles
Electrophiles
Energy depletion

→ Estimate MTD
Estimate NOEL

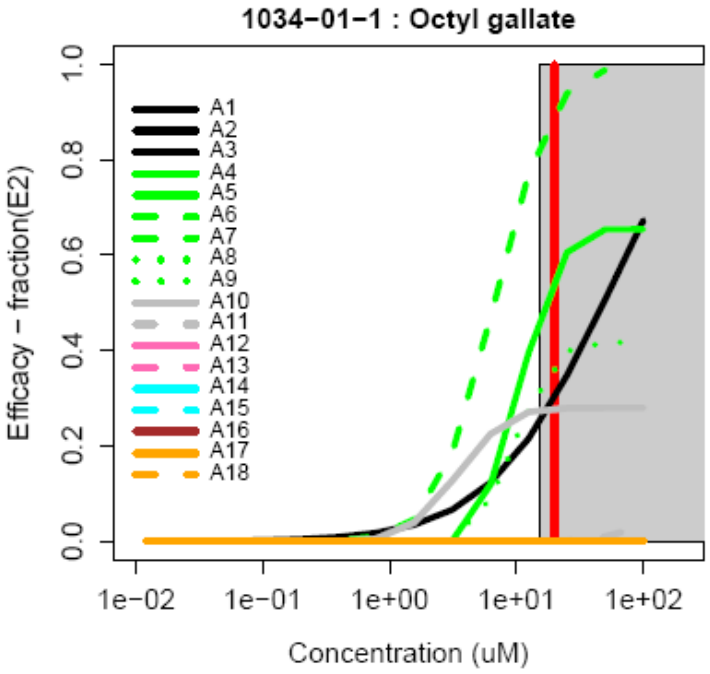
No Effect

Non-reactive chemical
Not bioactive
Effects would require high doses

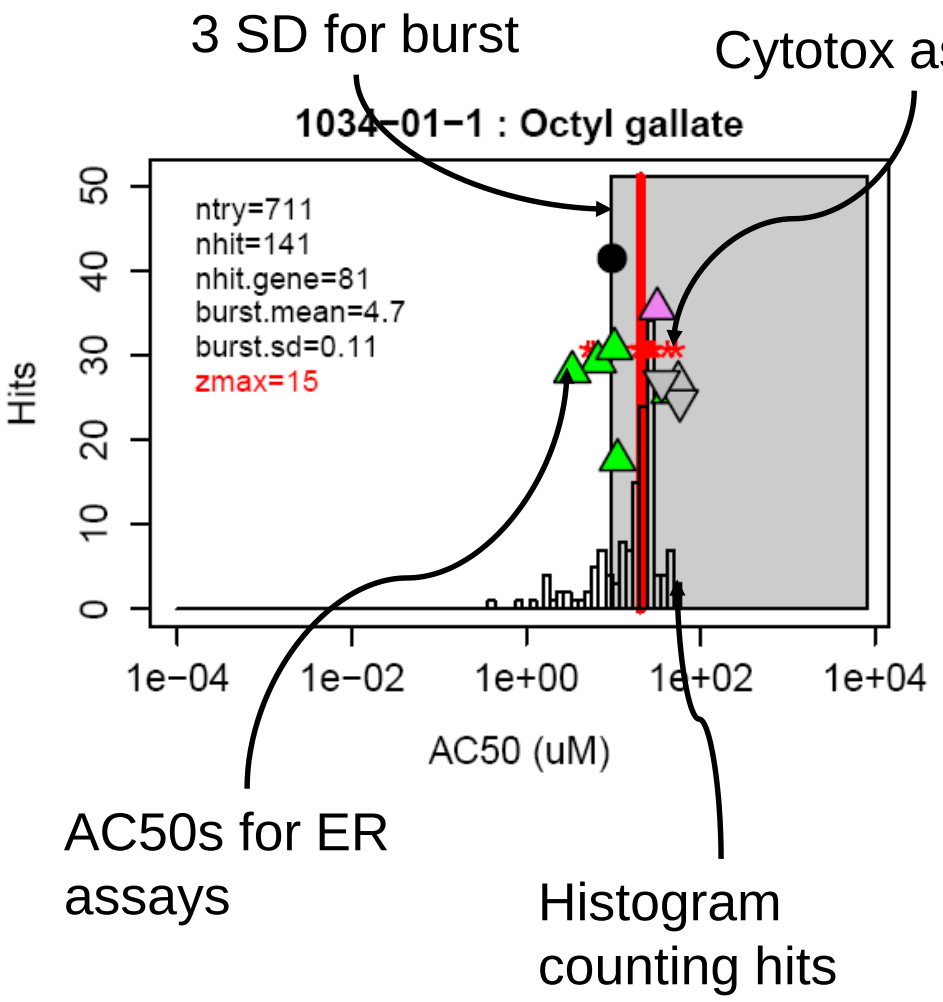
→ Estimate NOEL

Example illustrating assay data

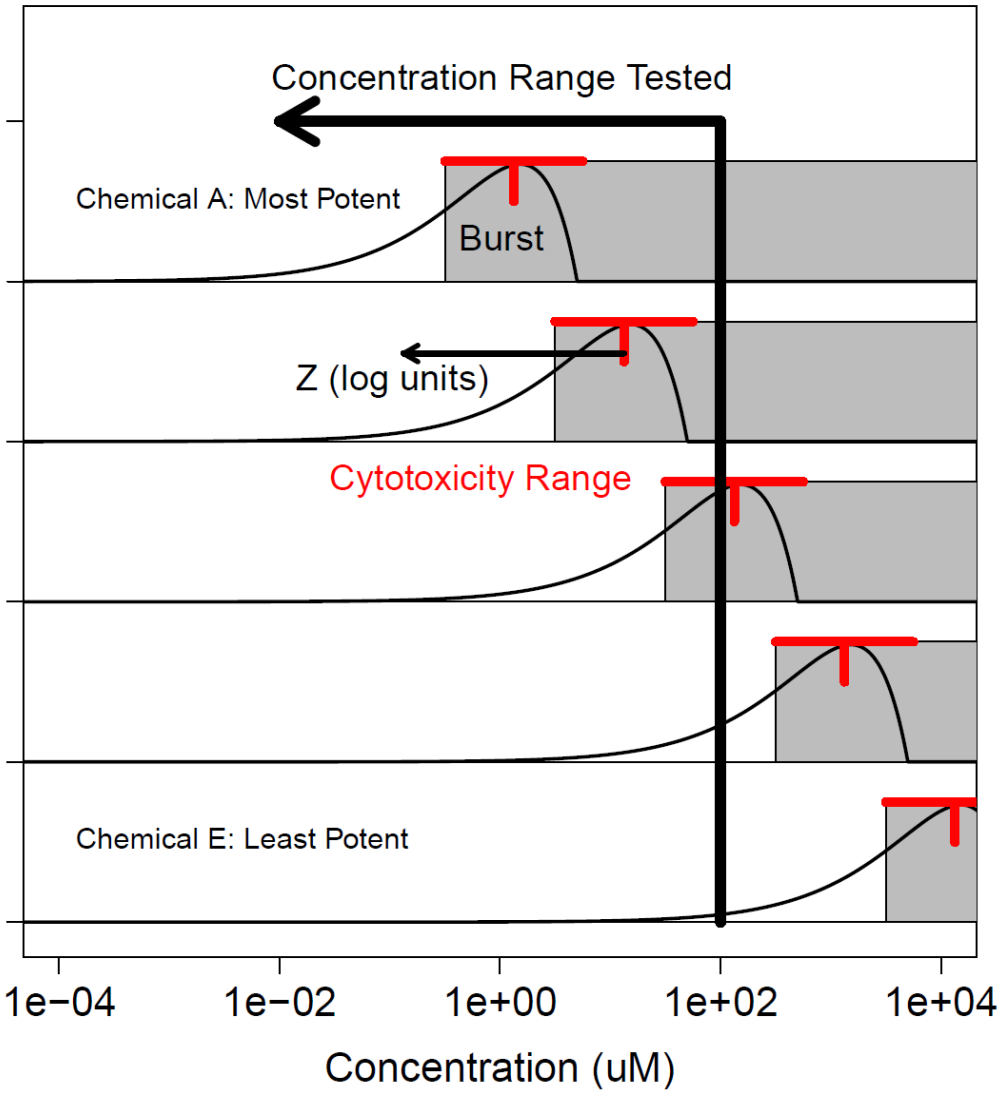
Concentration-response data for single gene (ESR1 / ER)



Histogram of AC50 Values



Most chemicals display a “burst” of activity at cell-stress or cytotoxicity concentration

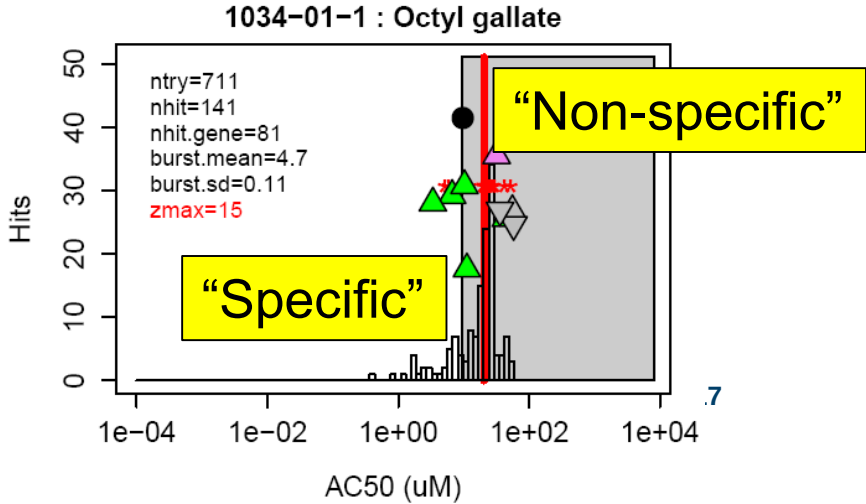


Most chemicals cause activity in many assays near the cytotoxicity threshold

Cell-stress related assay interference

“Hit” (AC50) in burst region is less likely to result from specific activity (e.g. binding to receptor or enzyme)

Z-score: # of SD from burst center
-High Z: more likely to be specific
-Low Z: less likely to be specific



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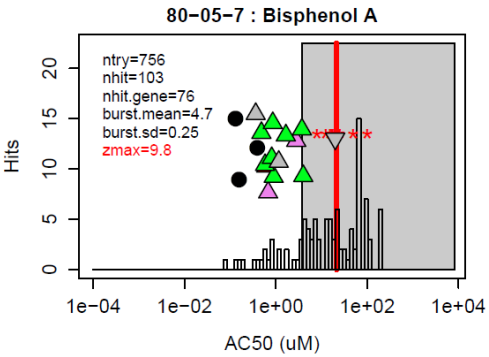

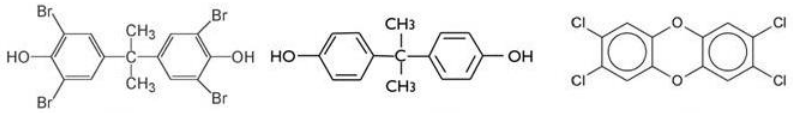

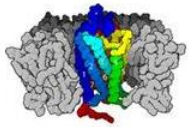

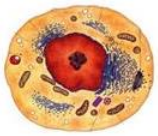

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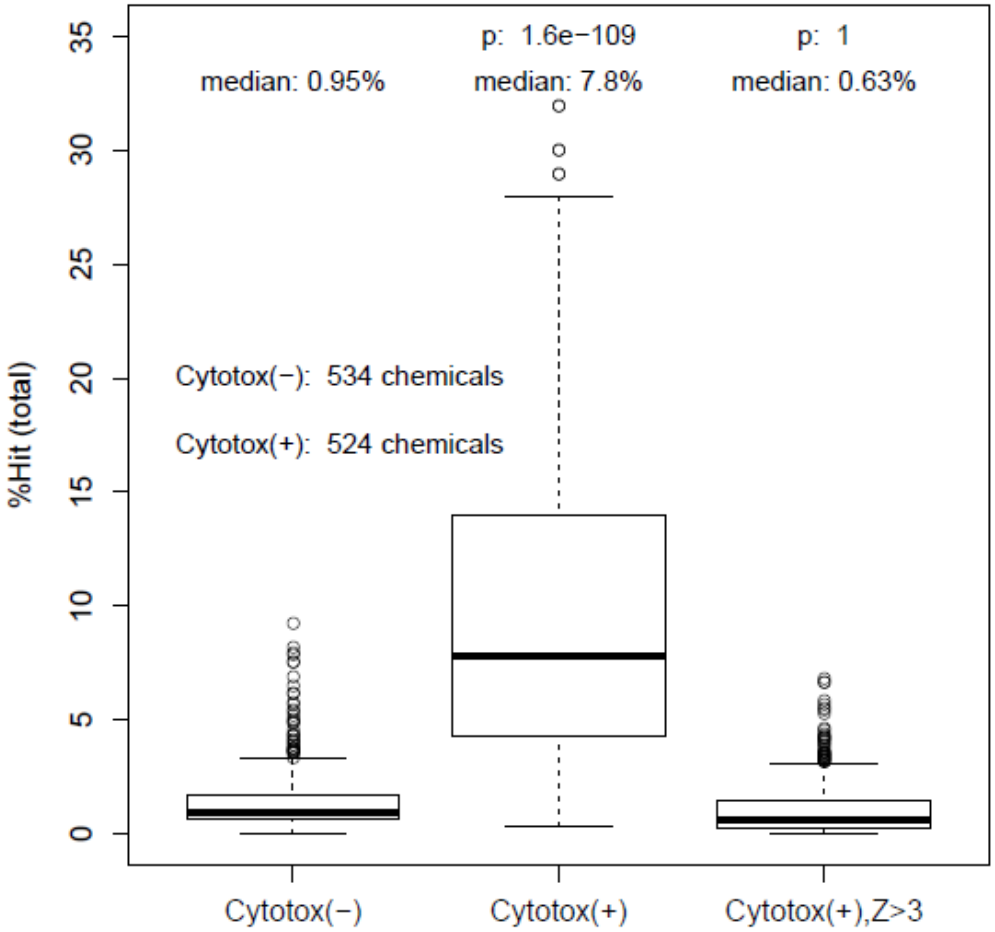
No Effect

Non-reactive chemical
Not bioactive
Effects would require high doses

→ Estimate NOEL

Non-specificity with cytotox is general

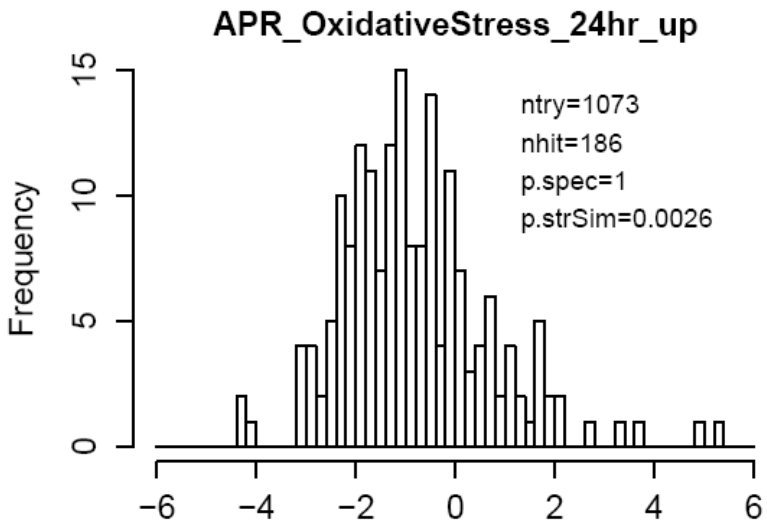


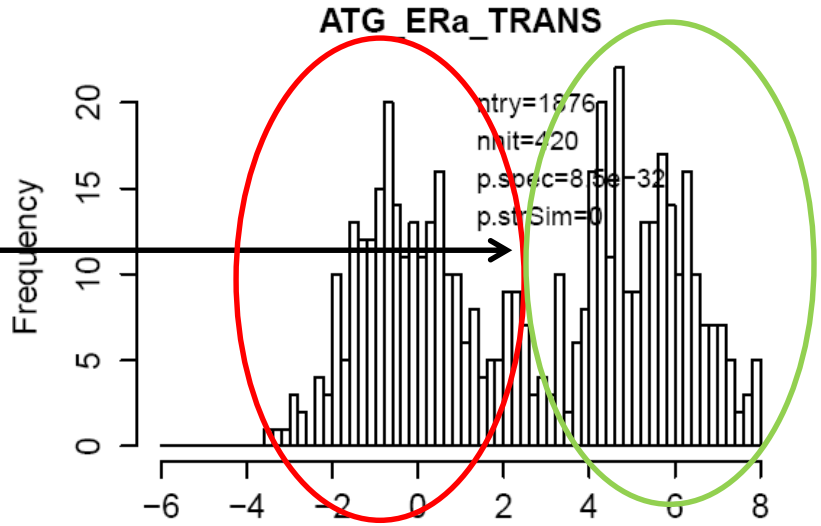
Having cytotoxicity @<100 uM greatly increases number of hits

Chemicals with cytotoxicity @<100 uM have many hits, but few are outside of burst

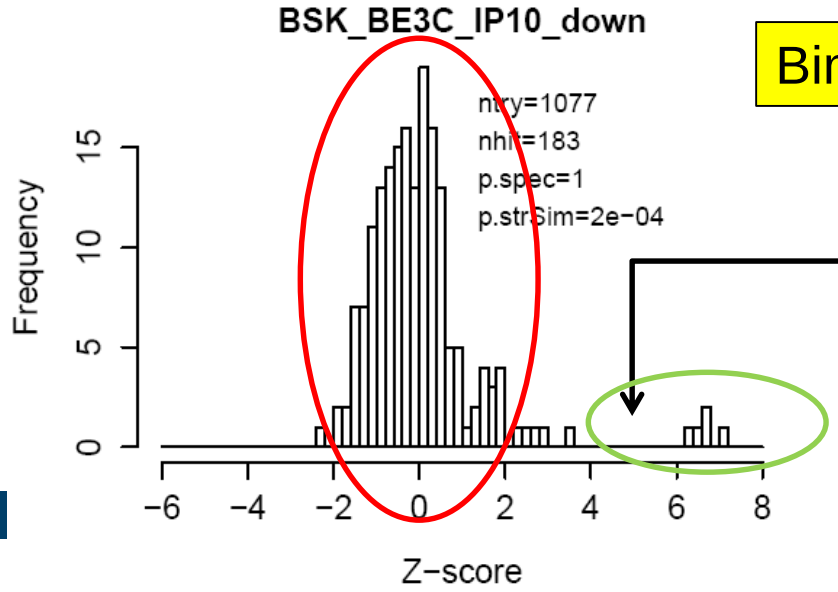
Examine Z-scores by assay

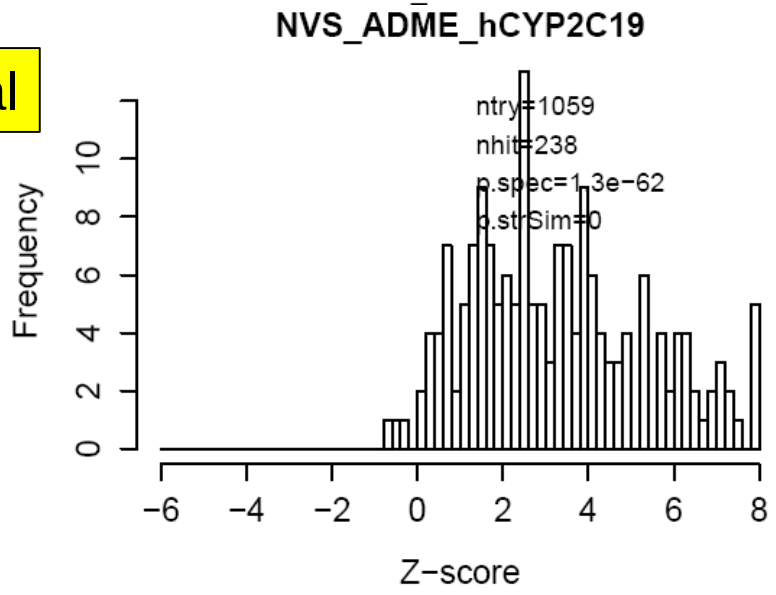
Cytotox / Cell Stress
"True" activity



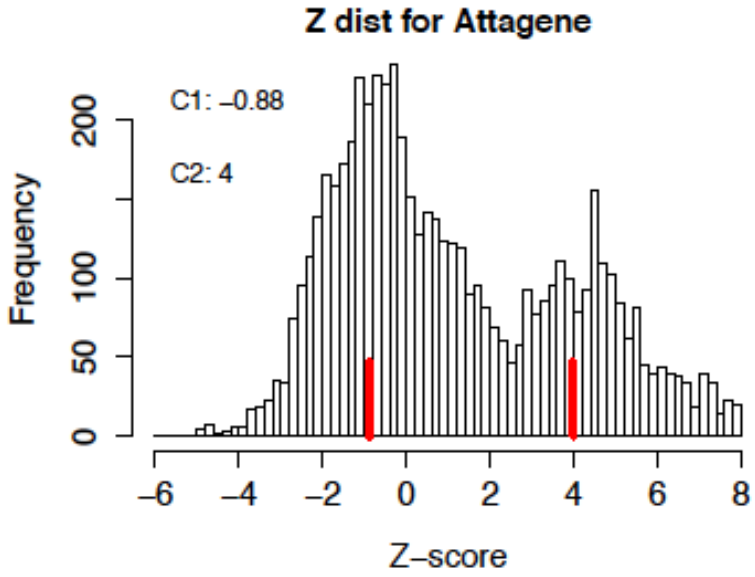


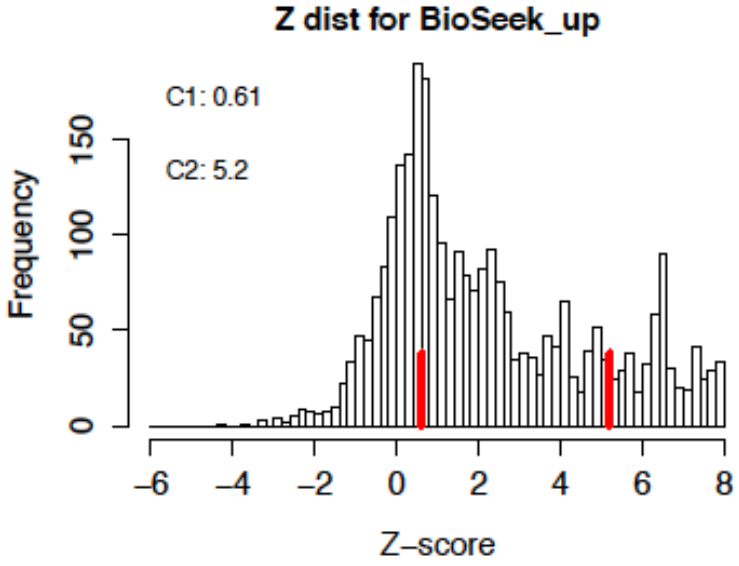
Bimodal

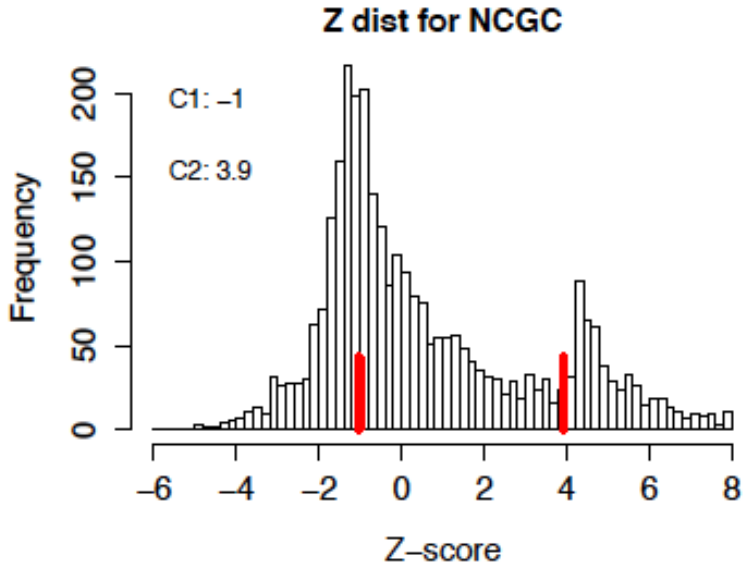




Bimodal distribution is general







Promiscuity: Highest for chemicals designed to be bioactive

Category	Nchem	Mean Hit Ratio	p-hot
conazole (triazoles)	13	0.034	3.5E-06
Pharma Class 4.86	10	0.031	1.1E-05
Pharma Class 4.58	11	0.029	4.1E-05
conazole (imidazoles)	6	0.031	0.003
Pharma Class 3.292	5	0.039	0.0049
steroid P	5	0.022	0.0052
Pharma Class 4.43	7	0.020	0.0067

Most Promiscuous Chemical Classes

2-3% of assays are active

All designed to be bioactive

Category	Nchem	Mean Hit Ratio	p-cold
alcohol primary	10	0.0011	0.00021
phthalate	17	0.0032	0.00084
carboxylate di	15	0.0028	0.0029
carboxylate	7	0.0015	0.0042

Least Promiscuous Chemical Classes

0.1-0.3% of assays are active

None designed to be bioactive

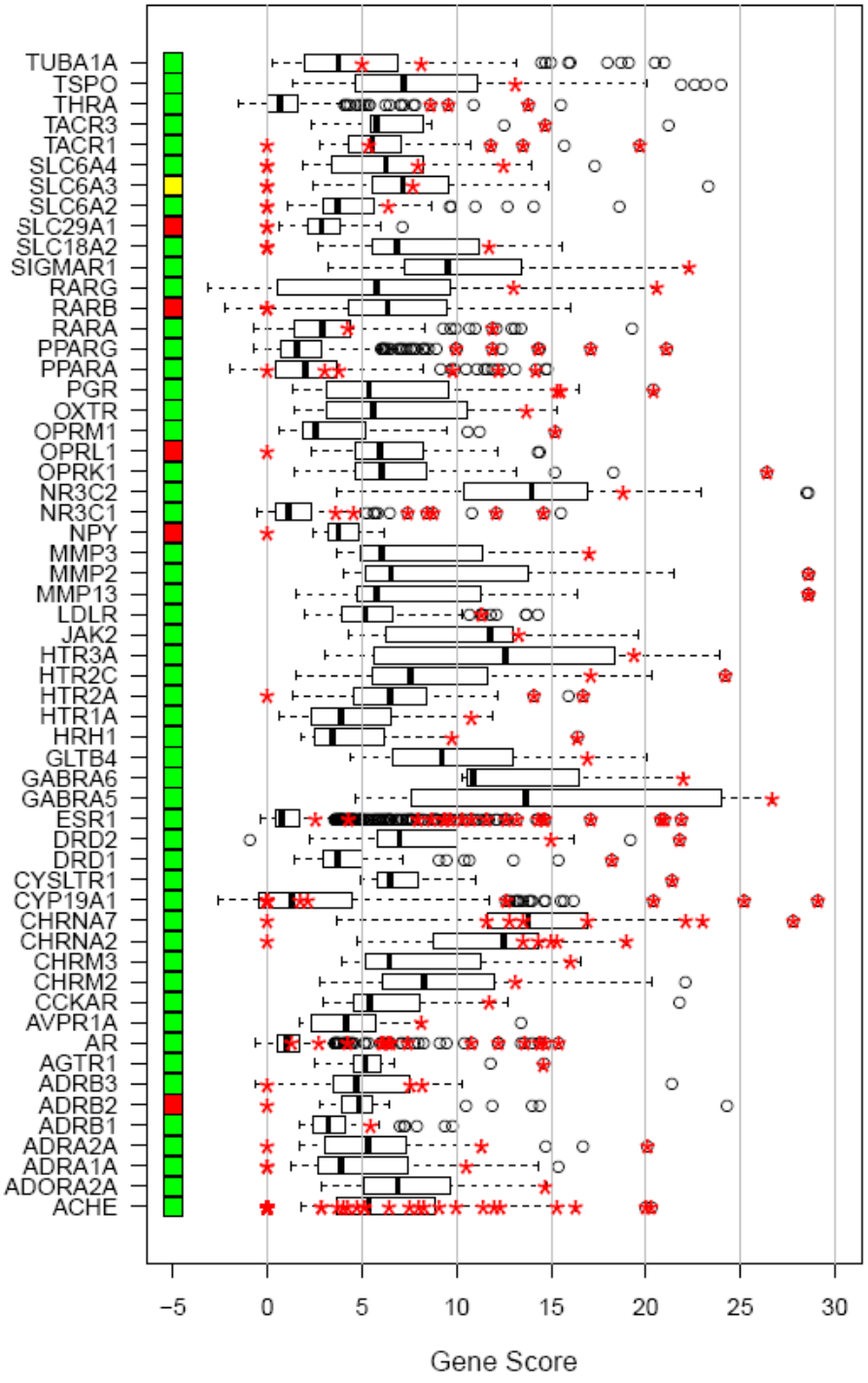
Do Assays Detect Potent Reference Chemicals?

* =Reference chemicals

- These chemicals should be near the right of the gene score distribution

- Most assays show reference chemicals to be potent and specific

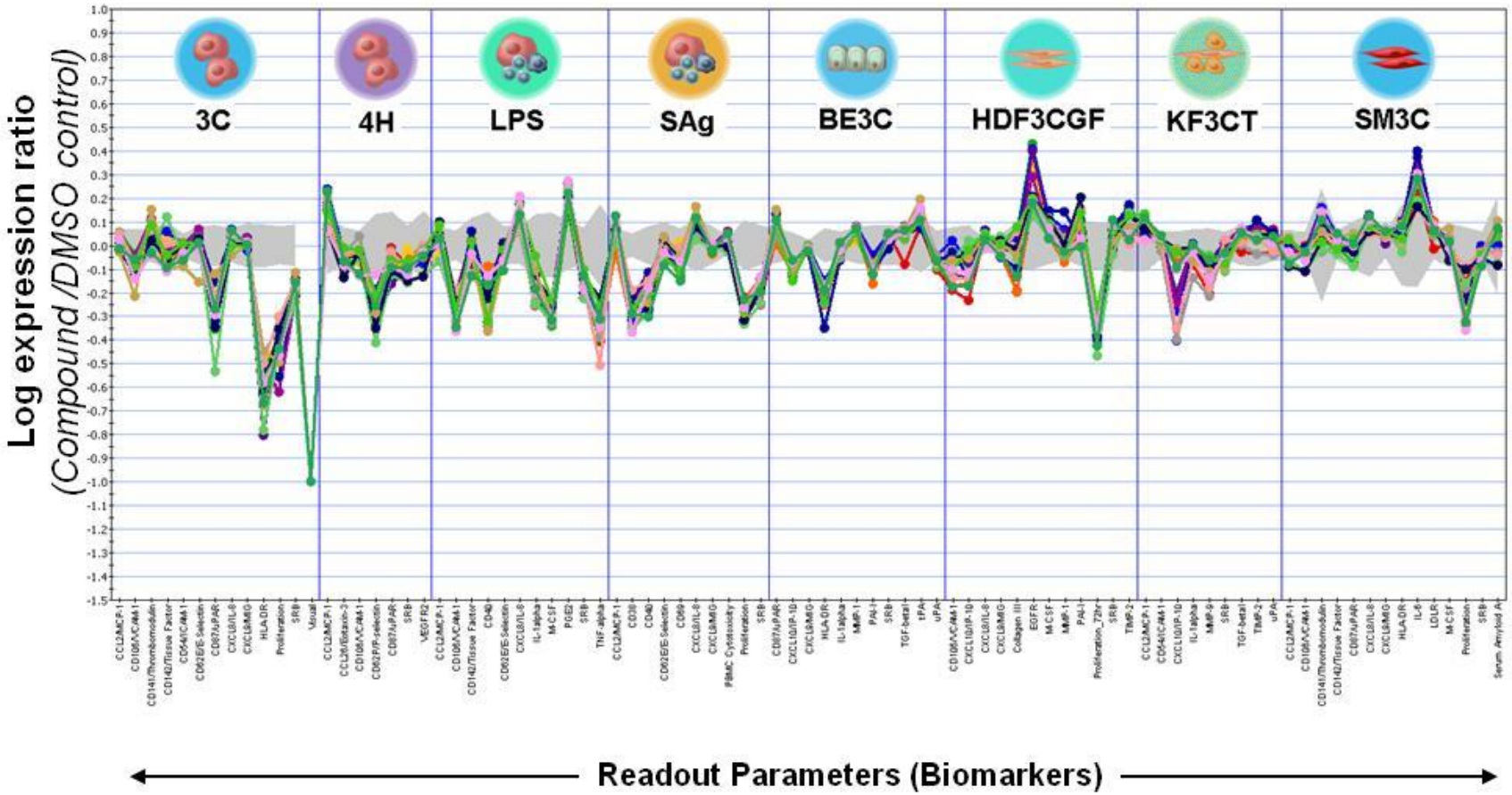
- Gives confidence that novel chemicals active in the assay are perturbing that pathway



Insights in to Mechanisms: BioMap Profiling Assays in Primary Human Cells

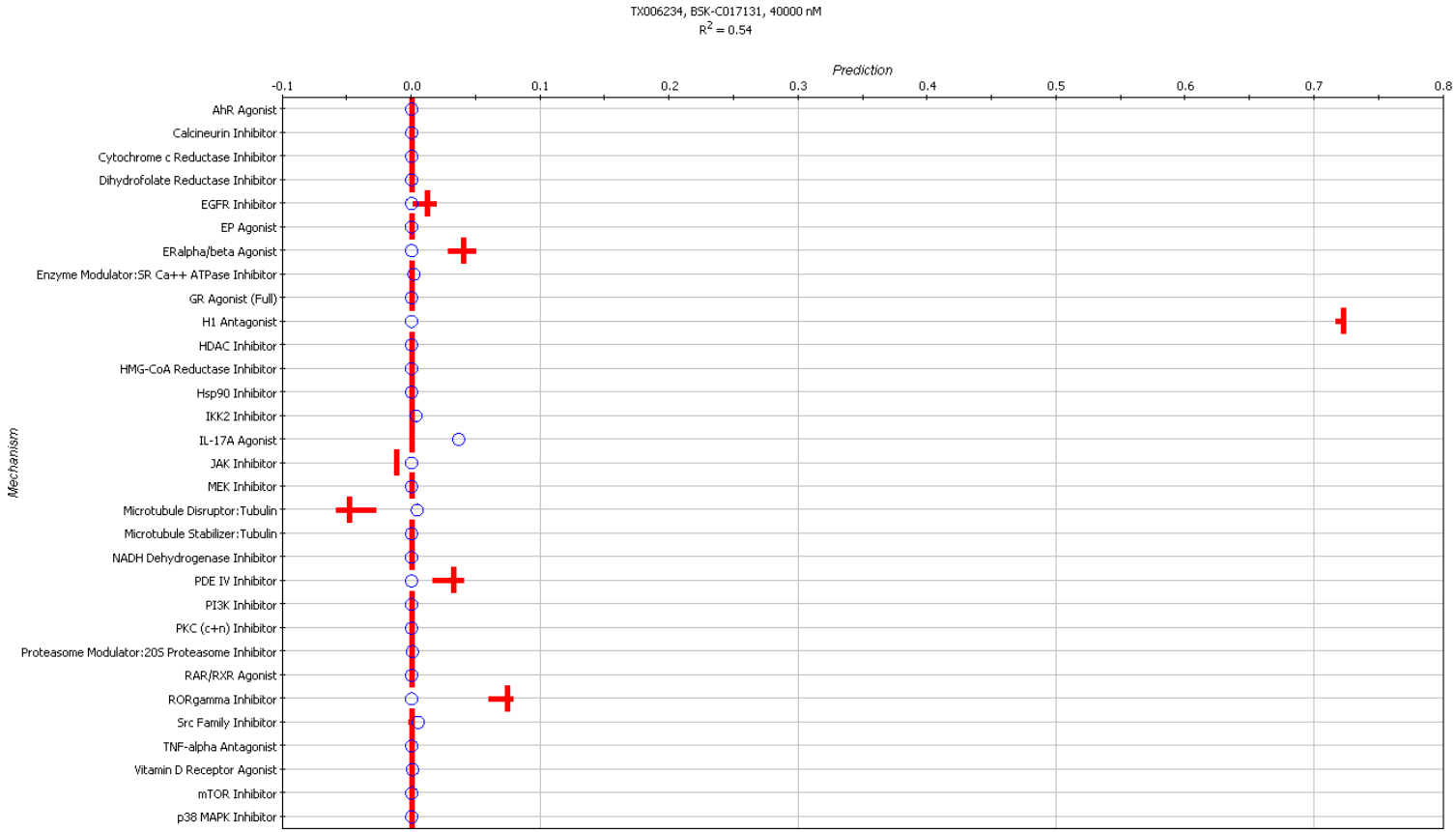
Table 1. Diversit

System
3C
4H
LPS
SAg
BE3C
HDF3CGF
KF3CT
CASM3C

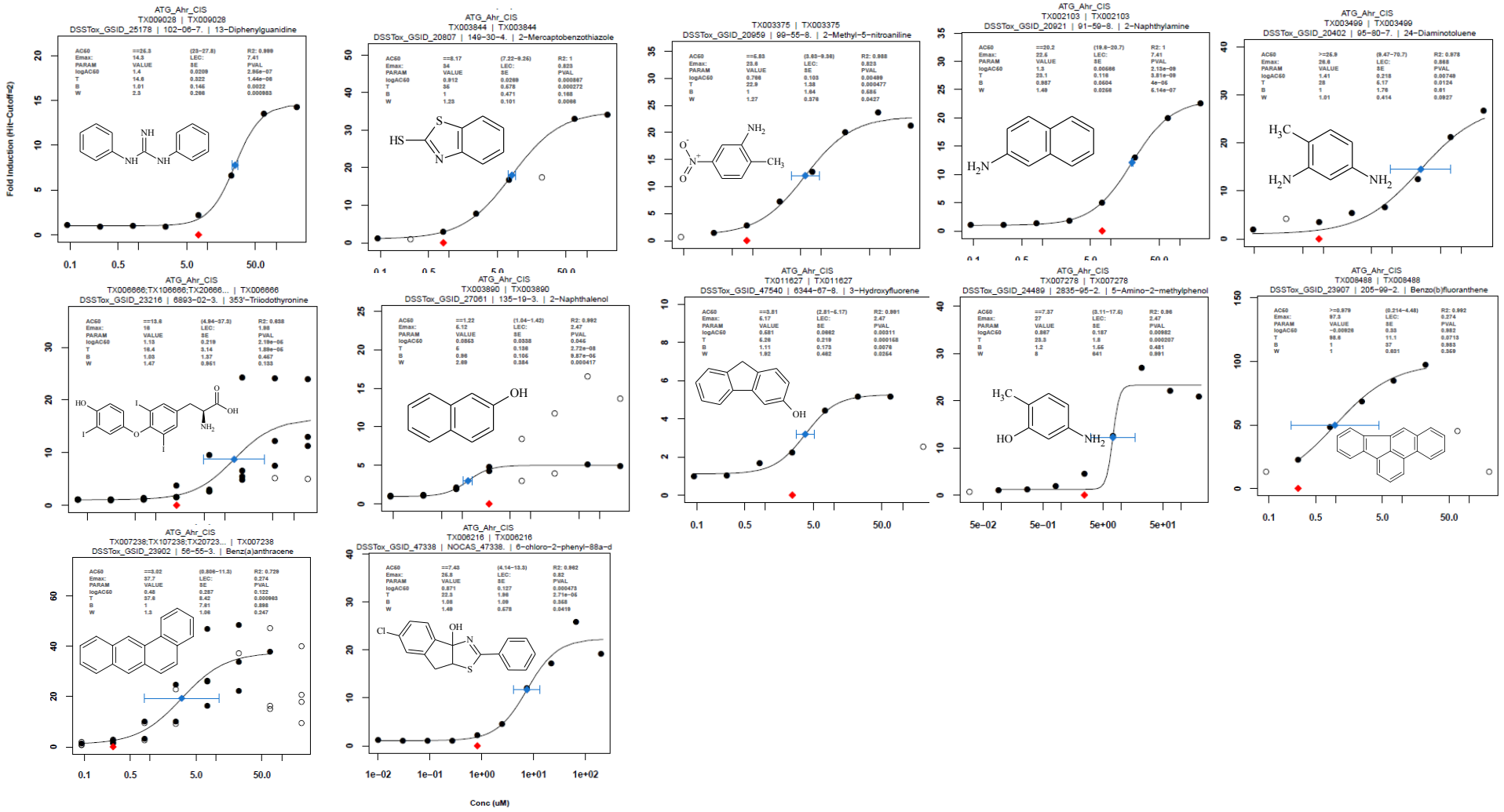


Mechanism Prediction: Supervised Classification by SVM

- Use Support Vector Machine learning algorithms
- Reference compounds are used at “clean” concentrations to build models (not ToxCast chemicals)
- 28 models built and applied to ToxCast data set

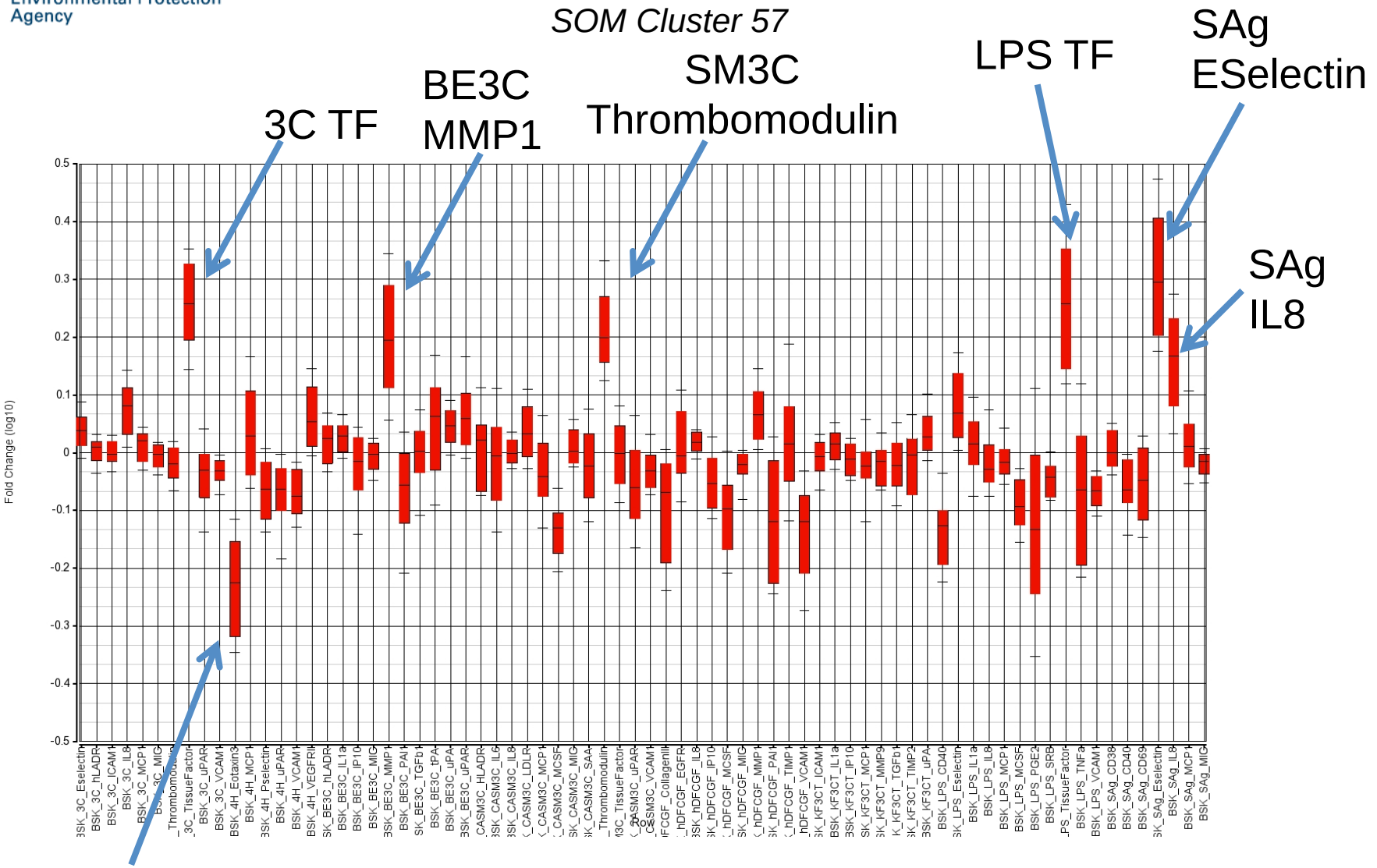


SVM Predictions of AHR Activity: Effects in ATG AHR Reporter Assay



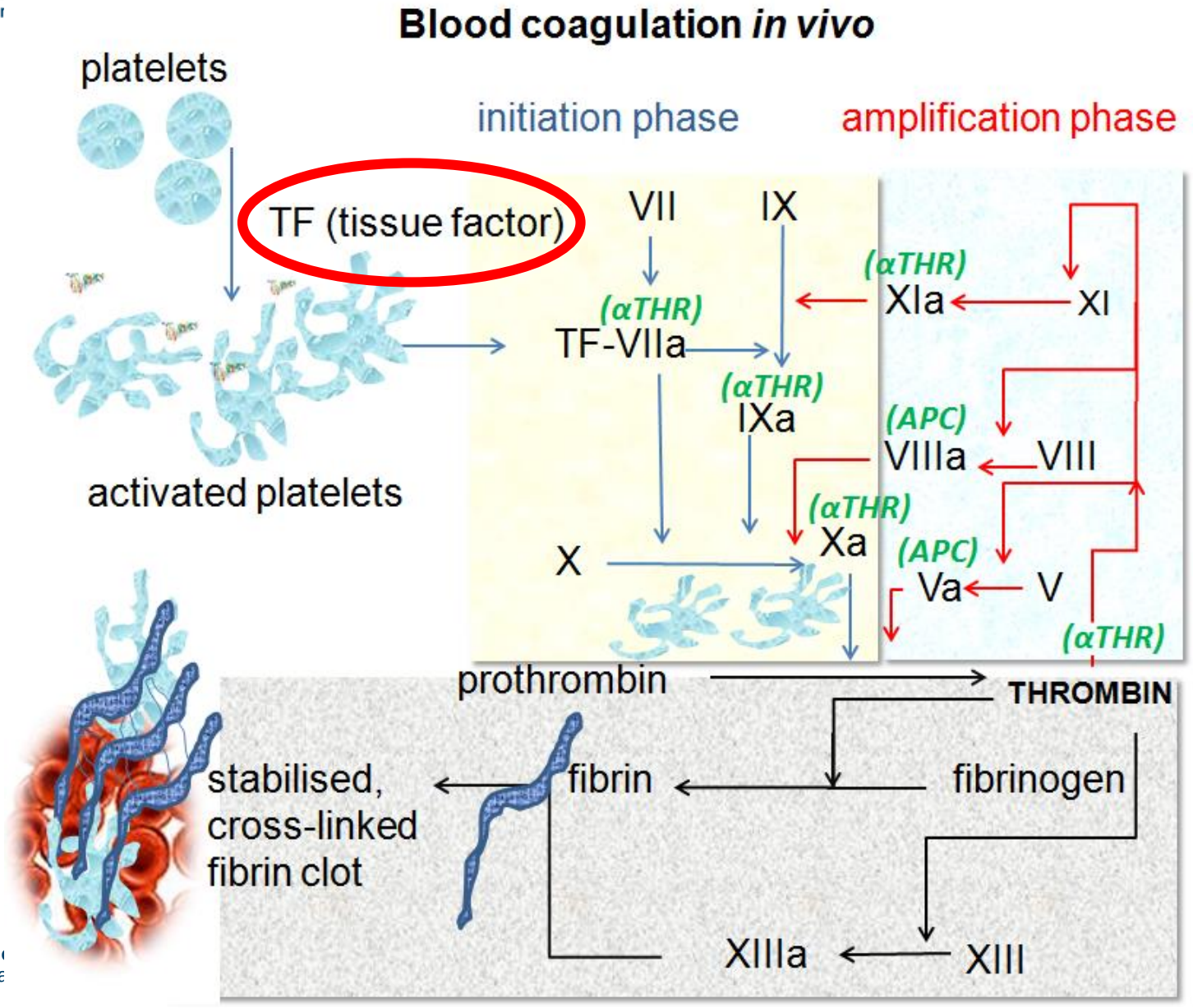
AHR Signature from Self Organizing Maps Analysis

SOM Cluster 57



PAHs from cigarette smoke associated with atherogenesis/thrombosis

Relationship of TF to Thrombosis



Dr Graham Beards, Wikipedia Commons

United States Environmental Protection Agency

Mitochondrial Toxicants SVM

Chemical	Mitochrome
Pharma	0.988
Azoxystrobin	0.987
Picoxystrobin	0.987
Azoxystrobin	0.986
Picoxystrobin	0.986
2-Methyl-4,6-dinitrophenol	0.984
Azoxystrobin	0.982
Picoxystrobin	0.981
2,4,5-Trichlorophenol	0.98
Triphenyl phosphate	0.98
Picoxystrobin	0.979
Azoxystrobin	0.974
Azoxystrobin	0.969
Fenofibrate	0.969
Dinoseb	0.967
Pharma	0.964
Pharma	0.954
Triclocarban	0.953
Zamifenacin	0.951
Dinoseb	0.946
Azoxystrobin	0.939
Nitrofen	0.939
Azoxystrobin	0.938
Phenolphthalein	0.933
Sodium azide	0.925
Progesterone	0.923
Triclocarban	0.917
Pentachlorophenol	0.916
Pharma	0.914
Triclosan	0.913

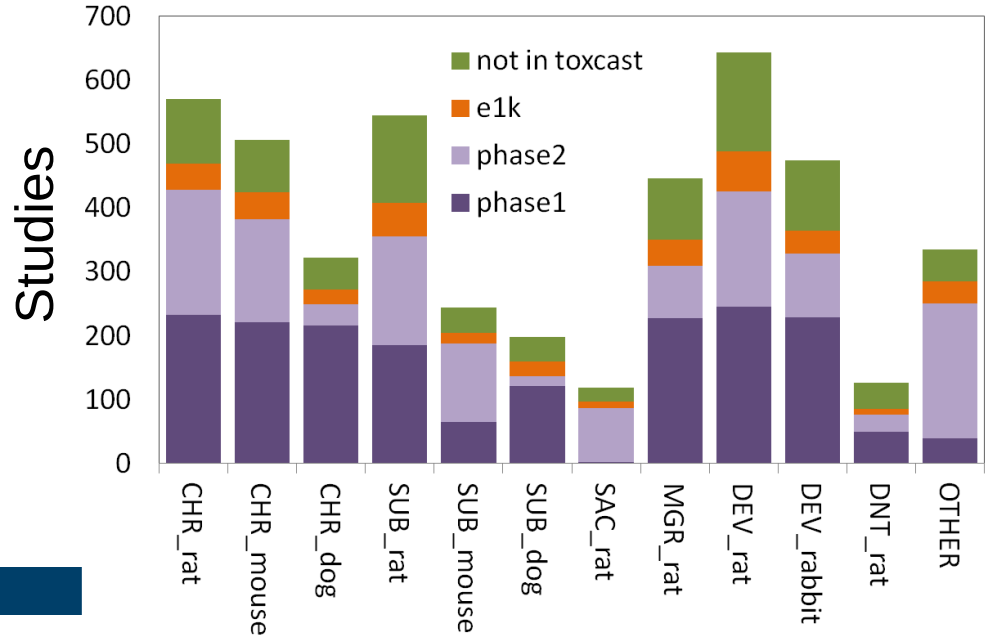
Predictive Models/Signatures

- Need to anchor to in vivo
- Guideline toxicity studies useful
  - EPA has extensive reports in support of registrations (pesticides)
  - Standardized
  - EPA regulates using these
- Recent incorporation of failed human drugs will provide more human-relevant in vivo

Toxicity Reference Database (ToxRefDB)

United States Environmental Protection Agency

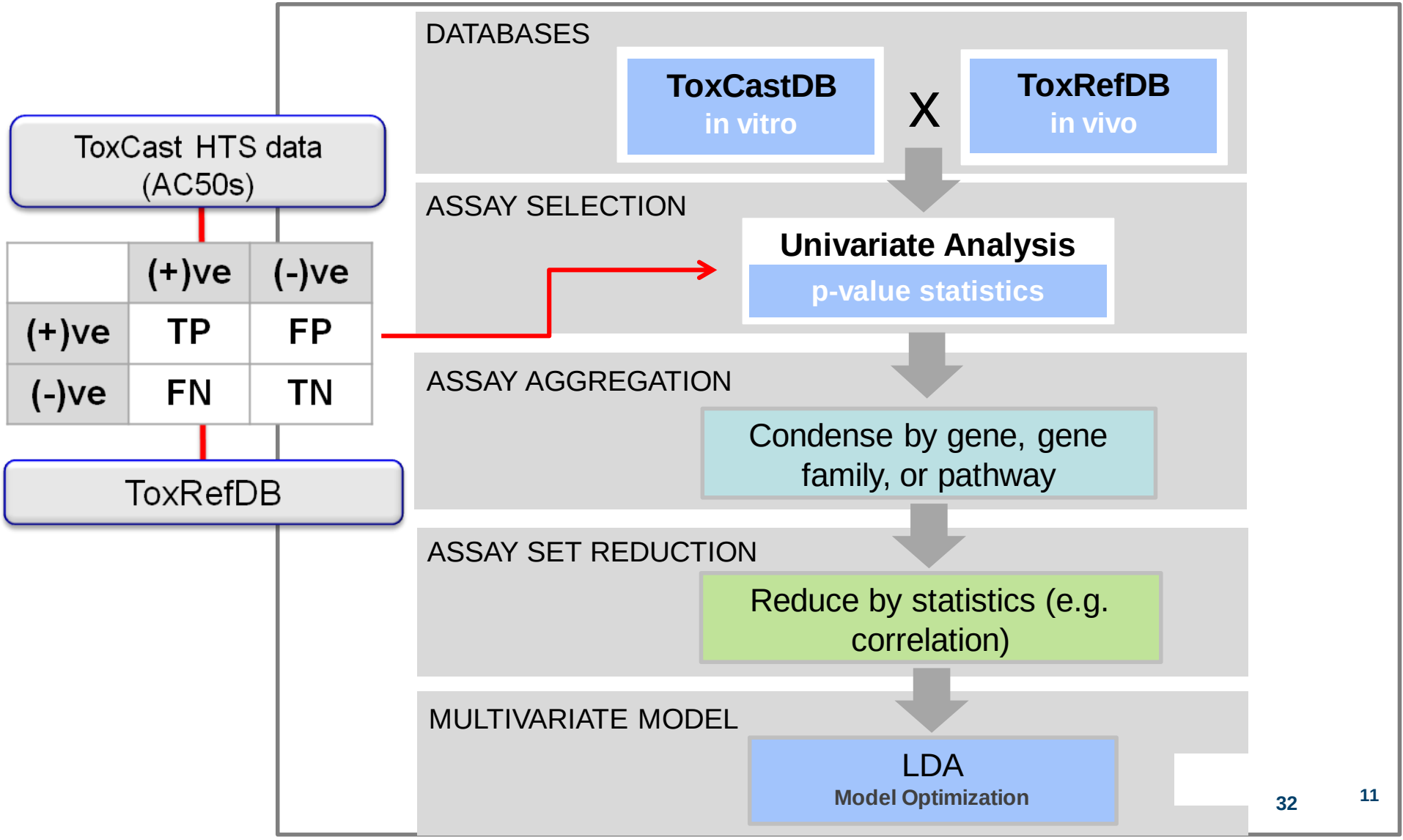
- ToxRefDB holds in vivo endpoint data from animal toxicology studies (DERs, NTP, open literature, pharma)
- Currently at 5567 studies on 1049 unique chemicals
- Used by:
  - ORD in predictive modeling (prospective)
    - e.g., multigen reproductive effects Martin et al., 2009)
  - OPP & OECD for assessing the impact of guideline studies on risk assessments (retrospective)
  - Public as a general chemical toxicity data resource



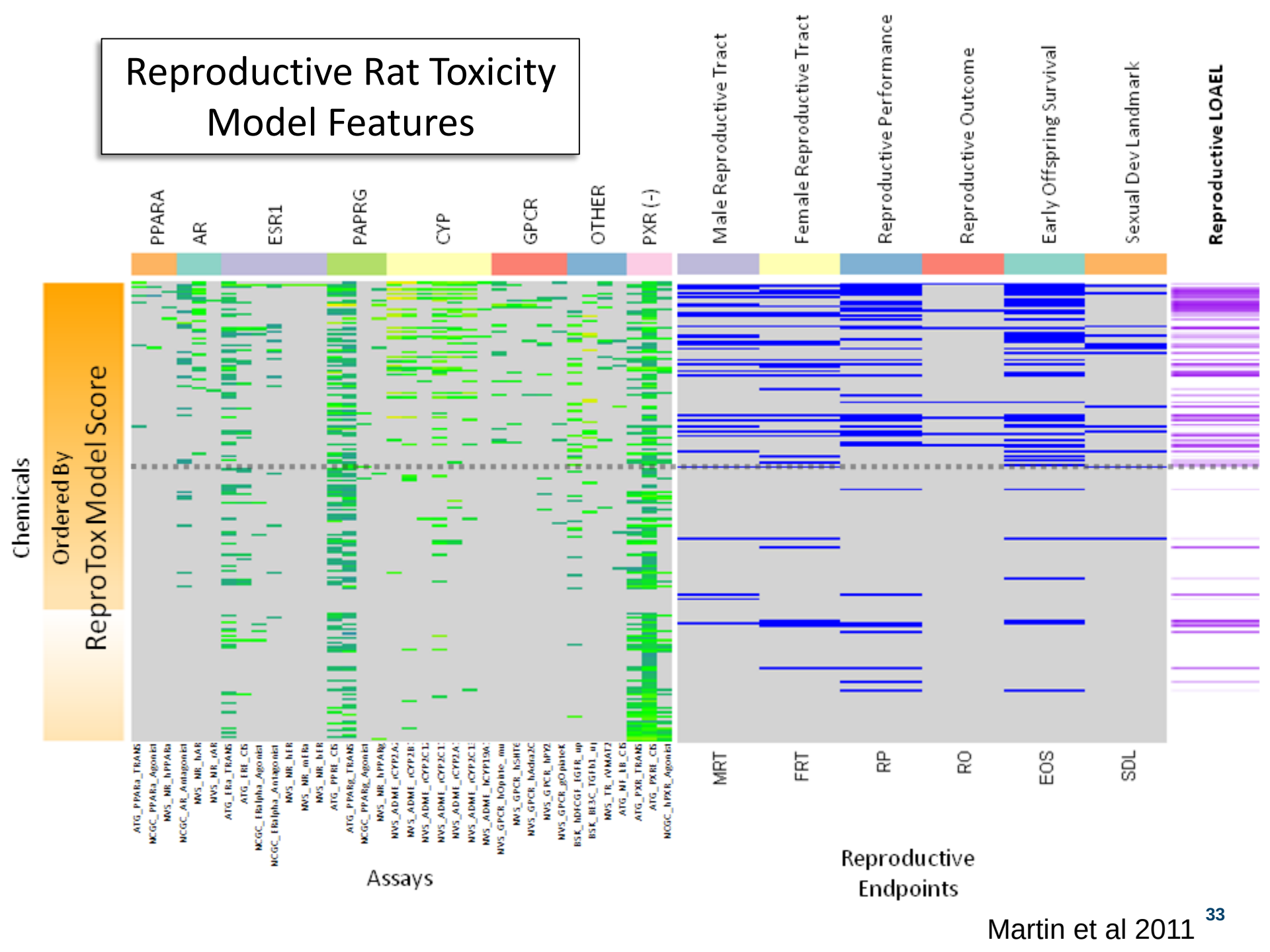
Data Source	Study Count
EPA OPP_der	3279
Open Literature	731
National Toxicol Program	666
Sanofi_pharma	222
Unpublished_submissions	50
GSK_pharma	38
Health Canada PMRA_der	23

http://actor.epa.gov/toxrefdb/

Predictive Model Development from ToxCast and Other Data

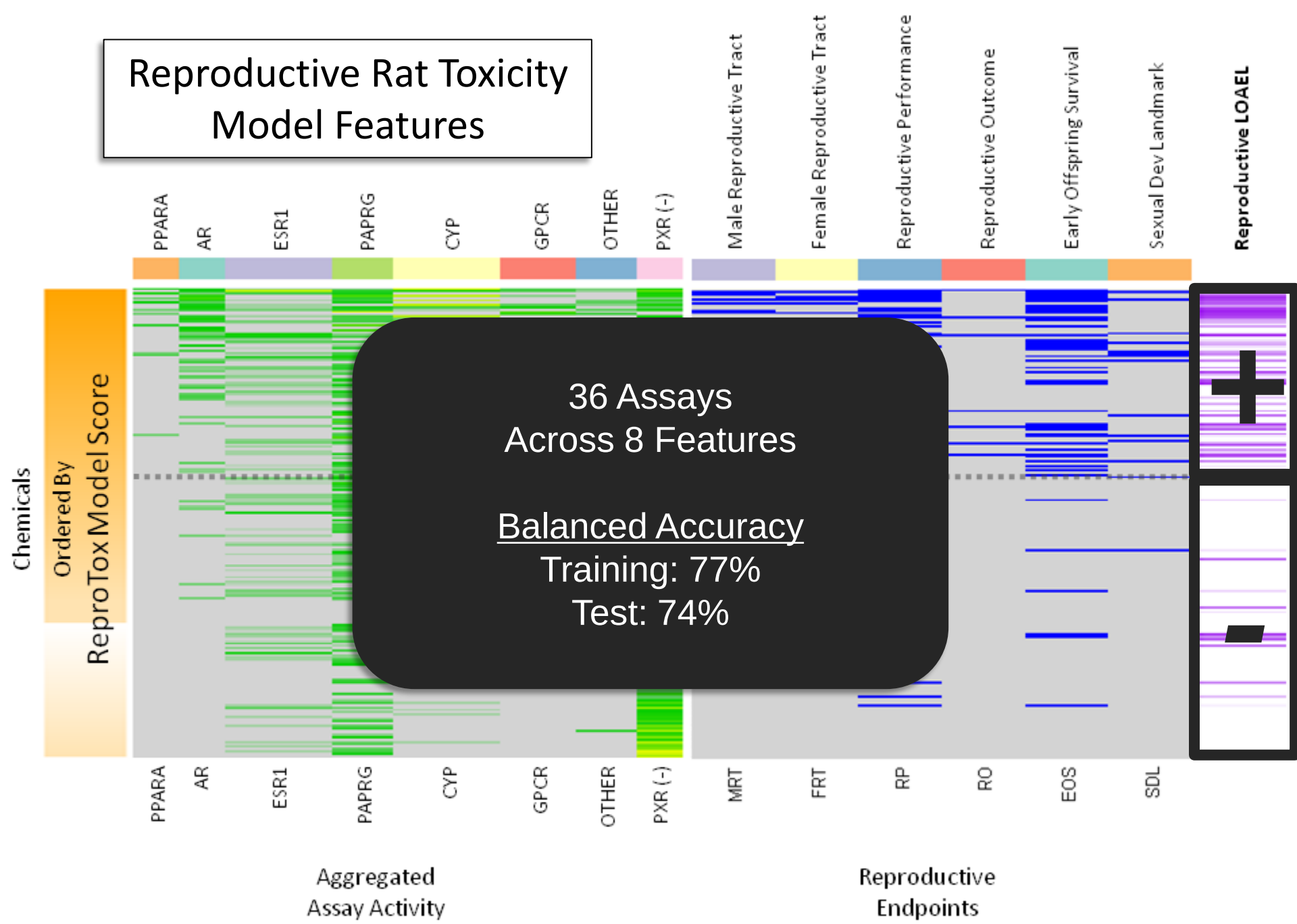


Reproductive Rat Toxicity Model Features



Martin et al 2011

Reproductive Rat Toxicity Model Features



36 Assays
Across 8 Features

Balanced Accuracy
Training: 77%
Test: 74%

Martin et al 2011

Predictive Toxicity Modeling Based on ToxCast Data

- **Predictive models: endpoints**
  - liver tumors: Judson et al. 2010, Env Hlth Persp 118: 485-492
  - hepatocarcinogenesis: Shah et al. 2011, PLoS One 6(2): e14584
  - cancer: Kleinstreuer et al. 2012, Toxicol Sci. 131:40-55
  - rat fertility: Martin et al. 2011, Biol Reprod 85: 327-339
  - rat-rabbit prenatal devtox: Sipes et al. 2011, Toxicol Sci 124: 109-127
  - zebrafish vs ToxRefDB: Sipes et al. 2011, Birth Defects Res C 93: 256-267

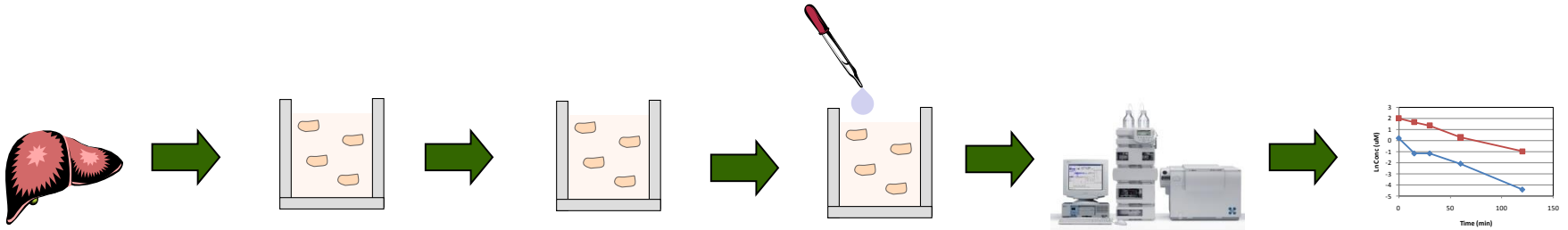
- **Predictive models: pathways**
  - endocrine disruption: Reif et al. 2010, Env Hlth Persp 118: 1714-1720
  - microdosimetry: Wambaugh and Shah 2010, PLoS Comp Biol 6: e1000756
  - mESC differentiation: Chandler et al. 2011, PLoS One 6(6): e18540
  - HTP risk assessment: Judson et al. 2011, Chem Res Toxicol 24: 451-462
  - angiogenesis: Kleinstreuer et al. 2011, Env Hlth Persp 119: 1596-1603
- **Continuing To Expand & Validate Prediction Models**
- **Generally moving towards more mechanistic/AOP-based models**

What dose will activate the pathway in vivo?

- Use Reverse Toxicokinetics approach (RTK)
  - Led by R. Thomas, B. Wetmore Hamner Inst.
  - Intrinsic clearance in human hepatocytes
  - Human plasma protein binding
- Integrate using one-compartment PK model
  - Yields C_{ss}/DR (concentration at steady state)
  - Units of μM/(mg/kg/day)
- RTK (SimCyp) provides estimates of population variability
  - Need to add estimates of uncertainty
- Use human cell lines and proteins

Rotroff, Wetmore, et al., Incorporating Human Dosimetry and Exposure into High-Throughput In Vitro Toxicity Screening. *Toxicol Sci* **2010,** *In press: doi:10.1093/toxsci/kfq220*.

Experimental Assays for Characterizing Steady-State Pharmacokinetics



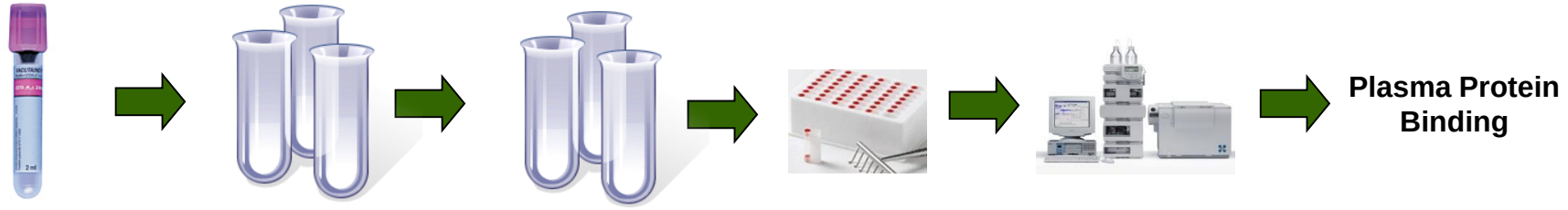
Human Hepatocytes (10 donor pool)

Add Chemical (1 and 10 μM)

Remove Aliquots at 15, 30, 60, 120 min

Analytical Chemistry

Hepatic Clearance



Human Plasma (6 donor pool)

Add Chemical (1 and 10 μM)

Equilibrium Dialysis

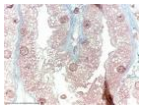
Analytical Chemistry

Plasma Protein Binding

Data Collected at Hamner Institutes

Pharmacodynamics

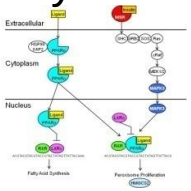
Adverse Effect



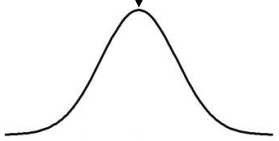
MOA

Key Events

Toxicity Pathway



HTS Assays



Biological Pathway Activating Concentration (BPAC) Probability Distribution

BPADL

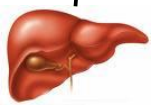
Probability Distribution for Dose that Activates Biological Pathway

Pharmacokinetics

Dose-to-Concentration Scaling Function (C_{ss}/DR) Probability Distribution

Populations

PK Model

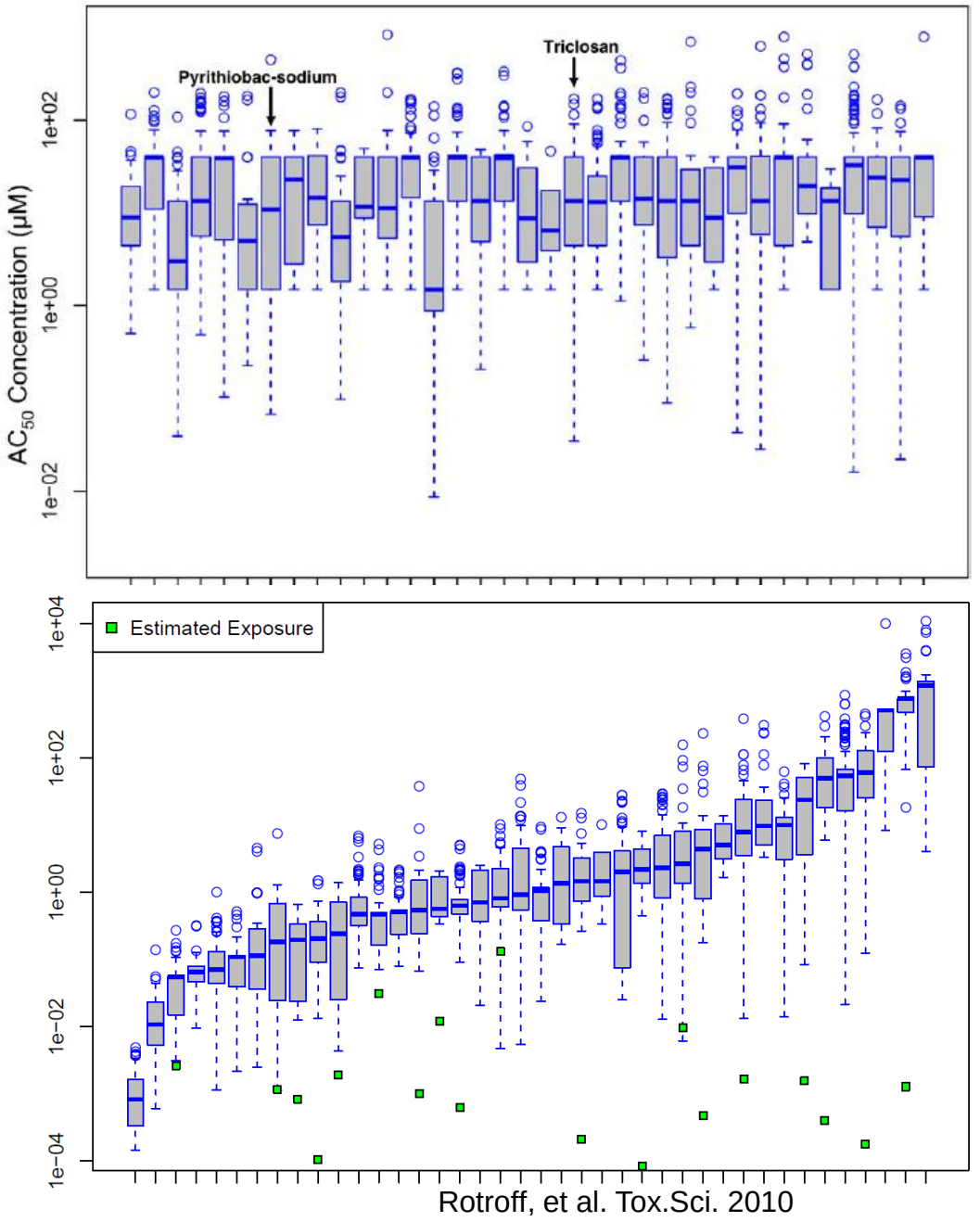


Intrinsic Clearance

Plasma Protein Binding

United States Environmental Protection Agency

The Significance of Reverse Toxicokinetics: Adding Kinetics is Critical to Understanding Dynamics



Rotroff, et al. Tox.Sci. 2010

Understanding Success and Failure

- Why *In vitro* to *in vivo* can work:
  - Chemicals cause effects through direct molecular interactions that we can measure with *in vitro* assays

- Why *in vitro* to *in vivo* does not always work:
  - Pharmacokinetics issues: biotransformation, clearance (**FP**, **FN**)
  - Assay coverage: don't have all the right assays (**FN**)
  - Tissue issues: may need multi-cellular networks and physiological signaling (**FN**)
  - Statistical power issues: need enough chemicals acting through a given MOA to be able to build and test model (**FN**)
  - Homeostasis: A multi-cellular system may adapt to initial insult (**FP**)
  - *In vitro* assays are not perfect! (**FP**, **FN**)
  - *In vivo* rodent data is not perfect! (**FP**, **FN**)

Summary

- Goal: use *in vitro* assays to screen and prioritize many data-poor chemicals
- Signature generation uses combination of biological insight and statistics
- Initial models point the way to real-world applications
- Further refinements are in the works
  - More chemicals and assays
  - Use of chemoinformatics
  - Systems-level models (vTissues)
  - Targeted testing approaches

EPA National Center for Computational Toxicology Staff
February 5, 2013